

mM EDTA and 0.05% marker dyes. Samples were heated to 75°C for 2 minutes immediately before electrophoresis through a 10% acrylamide gel (19:1 cross-linked), with 7 M urea, in a buffer of 45 mM Tris-Borate, pH 8.3, 1.4 mM EDTA. The expected product in reactions 1, 2, 5, and 6 is 85 nucleotides long; in reactions 3 and 5, the expected product is 27 nucleotides long. Reactions 4 and 8 were performed without pilot, and should remain at 206 nucleotides. The faint band seen at 24 nucleotides is residual end-labeled primer from the PCR.

The surprising result is that Cleavase® BB under these conditions causes all of the label to appear in a very small species, suggesting the possibility that the enzyme completely hydrolyzed the substrate. To determine the composition of the fastest-migrating band seen in lanes 5-8 (reactions performed with the deletion mutant), samples of the 206 base pair duplex were treated with either T7 gene 6 exonuclease (USB) or with calf intestine alkaline phosphatase (Promega), according to manufacturers' instructions, to produce either labeled mononucleotide (lane a of Figure 25B) or free  $^{32}\text{P}$ -labeled inorganic phosphate (lane b of Figure 25B), respectively. These products, along with the products seen in lane 7 of panel A were resolved by brief electrophoresis through a 20% acrylamide gel (19:1 cross-link), with 7 M urea, in a buffer of 45 mM Tris-Borate, pH 8.3, 1.4 mM EDTA. Cleavase® BB is thus capable of converting the substrate to mononucleotides.

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#### EXAMPLE 7

##### Nibbling Is Duplex Dependent

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The nibbling by Cleavase® BB is duplex dependent. In this example, internally labeled, single strands of the 206-mer were produced by 15 cycles of primer extension incorporating  $\alpha$ - $^{32}\text{P}$  labeled dCTP combined with all four unlabeled dNTPs, using an unlabeled 206-bp fragment as a template. Single and double stranded products were resolved by electrophoresis through a non-denaturing 6% polyacrylamide gel (29:1 cross-link) in a buffer of 45 mM Tris-Borate, pH 8.3, 1.4

mM EDTA, visualized by autoradiography, excised from the gel, eluted by passive diffusion, and concentrated by ethanol precipitation.

The cleavage reactions comprised 0.04 pmoles of substrate DNA, and 2  $\mu$ l of Cleavase® BB (in an *E. coli* extract as described above) in a total volume of 40  $\mu$ l of 5 10 mM Tris•Cl, pH 8.5, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>. Reactions were initiated by the addition of pre-warmed enzyme; 10  $\mu$ l aliquots were removed at 5, 10, 20, and 30 minutes, and transferred to prepared tubes containing 8  $\mu$ l of 95% formamide with 30 mM EDTA and 0.05% marker dyes. Samples were heated to 75°C for 2 minutes immediately before electrophoresis through a 10% acrylamide gel (19:1 cross-linked), with 7 M urea, in a buffer of 45 mM Tris•Borate, pH 8.3, 1.4 mM EDTA. Results 10 15 were visualized by autoradiography as shown in Figure 26. Clearly, the cleavage by Cleavase® BB depends on a duplex structure; no cleavage of the single strand structure is detected whereas cleavage of the 206-mer duplex is complete.

#### EXAMPLE 8

##### Nibbling Can Be Target Directed

The nibbling activity of the DNAPs of the present invention can be employed with success in a detection assay. One embodiment of such an assay is shown in Figure 27. In this assay, a labelled oligo is employed that is specific for a target sequence. The oligo is in excess of the target so that hybridization is rapid. In this 20 embodiment, the oligo contains two fluorescein labels whose proximity on the oligo causes their emission to be quenched. When the DNAP is permitted to nibble the oligo the labels separate and are detectable. The shortened duplex is destabilized and disassociates. Importantly, the target is now free to react with an intact labelled oligo. The reaction can continue until the desired level of detection is achieved. An 25 analogous, although different, type of cycling assay has been described employing lambda exonuclease. See C.G. Copley and C. Boot, *BioTechniques* 13:888 (1992).

The success of such an assay depends on specificity. In other words, the oligo must hybridize to the specific target. It is also preferred that the assay be sensitive;

the oligo ideally should be able to detect small amounts of target. Figure 28A shows a 5'-end  $^{32}\text{P}$ -labelled primer bound to a plasmid target sequence. In this case, the plasmid was pUC19 (commercially available) which was heat denatured by boiling two (2) minutes and then quick chilling. The primer is a 21-mer (SEQ ID NO:39). The 5 enzyme employed was Cleavase® BX (a dilution equivalent to  $5 \times 10^{-3}$   $\mu\text{l}$  extract) in 100 mM KCl, 10 mM Tris-Cl, pH 8.3, 2 mM MnCl<sub>2</sub>. The reaction was performed at 55°C for sixteen (16) hours with or without genomic background DNA (from chicken blood). The reaction was stopped by the addition of 8  $\mu\text{l}$  of 95% formamide with 20 mM EDTA and marker dyes.

10 The products of the reaction were resolved by PAGE (10% polyacrylamide, 19:1 cross link, 1 x TBE) as seen in Figure 28B. Lane "M" contains the labelled 21-mer. Lanes 1-3 contain no specific target, although Lanes 2 and 3 contain 100 ng and 200 ng of genomic DNA, respectively. Lanes 4, 5 and 6 all contain specific target 15 with either 0 ng, 100 ng or 200 ng of genomic DNA, respectively. It is clear that conversion to mononucleotides occurs in Lanes 4, 5 and 6 regardless of the presence or amount of background DNA. Thus, the nibbling can be target directed and specific.

#### EXAMPLE 9

##### Cleavase Purification

As noted above, expressed thermostable proteins, *i.e.*, the 5' nucleases, were 20 isolated by crude bacterial cell extracts. The precipitated *E. coli* proteins were then, along with other cell debris, removed by centrifugation. In this example, cells expressing the BN clone were cultured and collected (500 grams). For each gram (wet weight) of *E. coli*, 3 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 25 100 $\mu\text{M}$  NaCl) was added. The cells were lysed with 200  $\mu\text{g}/\text{ml}$  lysozyme at room temperature for 20 minutes. Thereafter deoxycholic acid was added to make a 0.2% final concentration and the mixture was incubated 15 minutes at room temperature.